Anomalous Nonidentity between *Salmonella* Genotoxicants and Rodent Carcinogens: Nongenotoxic Carcinogens and Genotoxic Noncarcinogens

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According to current data, the capacity to cause nonprogrammed or unscheduled cell proliferation in target tissues, a common characteristic of chemical carcinogens, may play a more important role in the development of tumors than does genotoxicity. This paper provides strong support for the validity of this conclusion. Ames-negative nongenotoxicants may be considered to be carcinogenic primarily because of their ability to induce cell proliferation in animal tissues and organs. In addition, such nongenotoxic carcinogens may also provide latent and modest DNA (equivocal) modifications that never lead to Ames-positive events. Conversely, noncarcinogenesis by Ames-positive agents is likely to be linked to a lack of stimulation of cell division. Nongenotoxic and genotoxic carcinogens rely on both cell proliferation and equivocal DNA modification for their full carcinogenicity. Such equivocal DNA modifications do not appear to be formed by tumor promoters. The role of cell proliferation may provide a favorable milieu for the occurrence of genetic instability, give rise to selective "apoptosis-resistant abnormal cells," and then affect clonal expansion of these cells. Therefore, understanding the influence of nongenotoxic and genotoxic carcinogens on cell proliferation capability is a key point in determining the mechanisms of chemical carcinogenesis. Considering the contradictory and common features of genotoxicants and carcinogens, early detection of nonprogrammed cell proliferation is the most effective approach to predict human and rodent carcinogenicity. *Key words*: Ames test, carcinogen, cell proliferation, genotoxic, hepatocarcinogen, 8-hydroxydeoxyguanosine, noncarcinogen, nongenotoxic, tumor promoters. *Environ Health Perspect* 104:40–46 (1996)

Enormous progress has been made within the past several decades in assessing human cancer risk from chemical carcinogens. In fact, attempts to estimate risk potential have given rise to contradictions between *Salmonella* genotoxicity and rodent carcinogenicity with the existence of nongenotoxic (Ames-negative) carcinogens and genotoxic (Ames-positive) noncarcinogens (*1–5*). The anomaly in question has provided us with a clue to interpretation of the relationship between genotoxicity and carcinogenicity. In terms of the cause of human carcinogenesis, the focus should now be placed on the role of nonprogrammed cell proliferation caused by exogenous agents rather than on their genotoxicity (*6–9*).

Preston-Martin et al. (*10*) claimed that human cancers are reflections of sustained cell proliferation caused by cell proliferative factors (consisting of chemical agents, hormones, etc.) because nondividing cells in adults such as nerve cells and cardiomyocytes never develop tumors. In addition, Croy (*11*) assumed that estimation of genotoxic effects alone does not provide an accurate assessment of cancer risk to humans from chemical exposure. At present, when newly developed chemicals give rise to Ames-positive events, they are almost always restricted from release into the human environment by government regulation. Ames-positive events however do not always equate with carcinogenic events. It is necessary therefore to detect carcinogenicity by examining each chemical's ability to stimulate cell proliferation.

More recently, Mason (*12*) and Okey et al. (*13*) reported that some polycyclic aromatic hydrocarbons, which are genotoxic carcinogens, have the capability to increase cell proliferation through dioxin–aromatic hydrocarbon (Ah) receptor ligand complexes. Data also suggest that cell proliferation increased by genotoxic carcinogens is closely related to the development of tumors. This is the basis for the studies presented in this paper, which question to what extent noncarcinogens, as well as nongenotoxic and genotoxic hepatocarcinogens, exert cell proliferative action on hepatocytes *in vivo* (*14–16*). The results show that most of the hepatocarcinogens tested clearly accelerate hepatocyte division whereas the majority of noncarcinogens gave no such effect.

The data suggest that the capacity to cause cell proliferation is common to nongenotoxic and genotoxic carcinogens. The mechanisms underlying this proliferation remain unclear in many cases, but the role of cell division in carcinogenesis is certainly a key point in the development of tumors. This paper reviews issues regarding nongenotoxic carcinogens and genotoxic noncarcinogens and proposes an interpretation in terms of nonprogrammed cell proliferation.

Definition of Nongenotoxic Carcinogens

The status of nongenotoxicants and genotoxicants should be evaluated using the standard Ames test alone, including a liver S9 mix from rats, mice, or hamsters. There are two principal reasons for this: 1) the standard Ames test has hitherto supplied a large number of the screening data on existing noncarcinogens as well as carcinogens, which provides the highest value of overall concordance, compared to other established genotoxicity tests (*1*); and 2) the simply defined terminology facilitates a general understanding to scientists studying mutation, cancer, and other fields. According to the definition of nongenotoxic carcinogens, at least 30% of existing carcinogens can be assigned to this category (*1–4*).

Jackson et al. (*17*) reported that almost all putative nongenotoxic carcinogens can be shown to be genotoxic when tested with a combination of several genotoxicity tests. The combined test system, however, also includes tests to detect tumor-promoting agents that have cell proliferative capabilities. Therefore, the data lead to questions about whether nongenotoxic carcinogens examined are indeed genotoxic.

In addition, almost all nongenotoxic carcinogens are also believed to induce genotoxicity in *Salmonella* TA102 (*18*), which is supersensitive to active oxygen production. Screening data obtained with TA102 have, however, been limited so far. Festing (*19*) indicated that F344 rats and B6C3F_1 mice are resistant to some genotoxicants in U.S. National Toxicology Program carcinogenesis bioassays and has argued for the necessity of a multistrain approach. Many scientists recognize that Ames tests are too sensitive to DNA damage to reliably estimate whether human and rodent carcinogenicity will actually result from long-term exposure.

Based on the definition of nongenotoxic carcinogens presented above, I believe that

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nongenotoxic carcinogens always result in cell proliferation and that most latent and modest DNA (equivocal) modifications never lead to Ames-positive events. Further details are presented below.

Cell Proliferation Caused by Nongenotoxic Carcinogens and Genotoxic Carcinogens

Most published reports have indicated that induction of carcinogenesis by nongenotoxic carcinogens depends on the capability to produce cell proliferation (*20–31*). There is, however, no absolute consensus because of the lack of adequate screening data on the exact relation between increased cell proliferation and nongenotoxic carcinogenicity (*32*). Therefore, I conducted a comprehensive screening program to examine whether nongenotoxic hepatocarcinogens cause an increase in cell proliferation after a single administration of each chemical to male F344 rats (9 weeks of age) or male $B6C3F_1$ (8 weeks of age) mice with the maximum tolerated dose (MTD) and one-half the MTD (*14–16*). After treatments at 24, 39, and 48 hr, hepatocytes were prepared by a collagenase-perfusion technique and then incubated for 4 hr in medium containing 370 kBq/ml of [methyl-^{3}H]thymidine.

The results are summarized in Table 1. In the replicative DNA synthesis (RDS) test, 52 of 63 nongenotoxic and genotoxic hepatocarcinogens clearly induced increased RDS events, whereas 24 of 31 genotoxic and nongenotoxic noncarcinogens did not cause such RDS increases. One example, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, was first reported as a hepatocyte RDS-negative chemical (*14*); however, it was subsequently found to give weak RDS-positive results (M. Miyagawa and Y. Uno, personal communications). The results showed that almost all hepatocarcinogens tested increased cell division, whether or not they belonged to nongenotoxic or genotoxic categories, indicating that the potential to cause proliferation is common to carcinogens regardless of whether they have genotoxic potency, as suggested by Cohen et al. (*33*).

The present RDS test has both strengths and weaknesses for predicting hepatocarcinogenicity of unknown chemicals. The strength of the test is linked to its short-term nature, and its weakness is the high doses required in comparison to the results of 2-year animal assays. Some samples showed false-positive or false-negative RDS events relative to their established hepatocarcinogenicity (Table 1). The false-positive RDS events may be principally caused by acute hepatotoxicity leading to regenerative cell proliferation when acute MTD levels are applied to animals. False-negative results may occur because exposure was performed by single gavage in the experiments. With longer-term exposure, such as in subacute and chronic toxicity experiments, eventual induction of drug-metabolizing enzymes appears to biotransform highly toxic intermediates. This would not be expected to occur with short-term exposure. The second cause of false negative results may be differences in drug distribution resulting from the use of a single treatment versus the long-term exposure in 2-year animal assays. Thus, the RDS approach may not give a perfect match for site, sex, or species in carcinogenicity. Similar considerations are applicable to any short-term experiments used to predict chemical carcinogenicity at the whole-body level. The present RDS data reveal that the test is extremely useful for early detection of nongenotoxic hepatocarcinogens.

In considering the potential of chemicals to stimulate cell proliferation, RDS

Table 1. Summary of hepatocyte replicative DNA synthesis (RDS) test results[a]

Chemical	RDS incidence (%) Rat	Mouse
Ames-negative hepatocarcinogens		
Aldrin		1.09
Acetaminophen	9.4	
11-Aminoundecanoic acid	–	
Barbital		0.96
Benzene		0.54
Benzofuran		1.41
Benzyl acetate		1.48
Carbon tetrachloride	14.9	
Chlordane		2.33
Chlorendic acid	–	–
Chlorobenzilate		1.13
Chlorodibromomethane		8.53
Chloroform	10.2	
5-Chloro-*o*-toluidine		1.15
Clofibrate	2.3	
p,p'-DDE		1.28
p,p'-DDT	1.3	0.95
Dehydroepiandrosterone	8.7	
p-Dichlorobenzene		1.87
Dicofol		–
Dieldrin		0.76
Di(2-ethylhexyl)adipate		0.58
Di(2-ethylhexyl)phthalate	2.6	1.60
Diethylstilbestrol	7.9	
p-Dioxane	1.4	
Dipyrone		–
Ethenzamide		–
D,L-Ethionine	–	
17α-Ethynylestradiol	9.5	
Furfural		1.43
Heptachlor		1.84
Hexachlorobenzene		0.66
α-Hexachlorocyclohexane	4.4	
Hexachloroethane		1.21
Hydroquinone		1.04
Methyl carbamate	1.1	
4,4'-Methylenebis(*N,N'*-dimethyl) benzenamine		0.73
N-Methylolacrylamide		–
Mirex		1.04
Pentachloroethane		2.78
Pentachlorophenol		1.01
Phenobarbital sodium	2.0	2.78
Phenylbutazone		–
Polybrominated biphenyls	4.5	
Tannic acid	12.7	
1,1,1,2-Tetrachloroethane		0.88
1,1,2,2-Tetrachloroethane		1.41
Tetrachloroethylene		0.60
Thioacetamide	1 3.8	
Triadimefon		0.88
Trichloroacetic acid		0.98
1,1,2-Trichloroethane		1.28
2,4,6-Trichlorophenol		–
Tris (2-ethylhexyl) phosphate		–
Wy-14,643	4.1	
Zearalenone		–
Ames-positive hepatocarcinogens		
2,4-Diaminotoluene	2.3	
Dimethylnitrosamine	10.8	
2-Methoxy-5-methylaniline	6.0	
2,4,5-Trimethylaniline	2.5	
Safrole	5.5	
Trichloroethylene	1.4	1.36
Urethane	13.2	
Ames-negative noncarcinogens		
o-Anthlanilic acid	–	–
L-Ascorbic acid	–	–
Benzoin	9.6	–
Benzyl alcohol	–	–
Butylated hydroxyltoluene	9.2	0.80
ε-Caprolactam	–	–
3-Chloro-*p*-toluidine	3.0	0.89
o-Dichlorobenzene	–	–
2,4-Dichlorophenol	–	–
EDTA trisodium	–	–
Geranyl acetate	5.7	0.88
Lindane		
Lithocholic acid	–	2.05
D-Mannitol	–	–
D,L-Menthol	6.0	1.69
Methoxychlor	–	–
Phenol	–	–
Piperonyl butoxide		–
Sulfisoxazole	–	–
Titanium dioxide		–
Toluene		–
L-Tryptophan	–	–
Ames-positive noncarcinogens		
2-Chloroethanol	–	–
2,5-Diaminotoluene H_2SO_4	–	–
2,6-Diaminotoluene	–	–
Dimethoate	–	–
8-Hydroxyquinoline	–	–
Methyl methacrylate		–
4-Nitroanthranilic acid	–	–
4- Nitro-*o*-phenylenediamine	–	0.85
p-Phenylenediamine 2HCl	–	–

[a]Incidences of 0.4% or more are judged as positive responses in the mouse RDS test, those of 1.0% or more are positive in the rat RDS test; – indicates negative response.

events can be classified into three categories: 1) binding to the steroid–thyroid–retinoic acid receptor, a type which is not related to liver injury, 2) growth factor-binding due to chemical injury to the liver or other organs, and 3) a result of tumor promoters that never cause equivocal DNA modifications.

The chemical-binding steroid receptor superfamily is known to include the steroid hormonelike receptor, the peroxisome proliferator-activated receptor (PPAR), and probably the Ah receptor (*34–37*). Each receptor might act as a ligand for some of the RDS-positive carcinogens listed in Table 1: a steroid hormonelike receptor for dehydroepiandrosterone and 17α-ethynylestradiol; the PPAR for dehydroepiandrosterone, clofibrate, di(2-ethylhexyl)adipate (DEHA), di(2-ethylhexyl)-phthalate (DEHP), phenobarbital sodium, tetrachloroethylene, trichloroethylene, and Wy-14,643; and the Ah receptor for benzo[*a*]pyrene, *p,p'*-DDT, *p,p'*-DDE, and polybrominated biphenyls (*34,36,37*).

Although there is no clear evidence for an increase in cell proliferation through the PPAR receptor (*37*), some proto-oncogenes promoting cell proliferation, e.g., *fos* and *jun*, are known to be activated (*34,38,39*). Our RDS data suggest that formation of PPAR–ligand complexes also leads to cell proliferation (Table 1). Thus, steroid-superfamily receptor-mediated cell proliferation can be considered to be principally involved in early development of hepatocyte RDS induction. In addition, ligands may simultaneously give rise to equivocal DNA modifications. Progression of both phenomena in the same cells may result in effective disruption of cell-cycle controls so that hepatocarcinomas eventually arise. Among these receptors, particular attention is now being paid to the Ah receptor because it can react with a wide range of nongenotoxic and genotoxic agents as a ligand and it principally regulates induction of CYP1A1 to metabolically activate polycyclic aromatic hydrocarbons (*13*).

Several growth factors are also known to increase cell proliferative events in specific cells in tissues (*40*), but the relationship between the roles of growth factors and cell proliferation in carcinogenesis is extremely difficult to assess. With regard to hepatocyte cell proliferation, the action of hepatocyte growth factor (HGF) investigated by Nakamura and his co-workers (*41,42*) must be considered: HGF secreted from liver and other organs in response to chemical injury is known to promote the division of hepatic parenchymal cells by means of paracrine/endocrine mechanisms; moreover, the nature of the HGF-binding receptor has also been identified as a *met* proto-oncogene product (*43*). Other growth factors taken into the liver are epidermal growth factor and transforming growth factor-ß1. At present, there is no simple explanation of how growth factors act in combination to bring about complicated events *in vivo*.

Of several RDS-positive carcinogens listed in Table 1, at least 13 samples are generally well known to be hepatotoxicants in rats and mice (*13,14*): aldrin, benzene, carbon tetrachloride, chloroform, pentachloroethane, pentachlorophenol, tannic acid, 1,1,1,2-tetrachloroethane, terachloroethylene, thioacetamide, trichloroacetic acid, trichloroethylene, and 1,1,2-trichloroethane. Thus, damage-induced HGF is likely to play an important role in hepatocyte RDS induction. In addition, the ligand may give rise simultaneously to equivocal DNA modifications, as in the case of the steroid-superfamily receptor. Interestingly, hepatocyte RDS events do not always directly reflect hepatotoxicity, either pathologically or biochemically (e.g., when HGF is secreted through paracrine mechanisms). Therefore, increased hepatocyte RDS events might not be due to simple hepatotoxicity to target population.

In vivo liver-tumor promoters such as butylated hydroxytoluene and lithocholic acid, which are classified as noncarcinogens, might cause increased hepatocyte RDS events by some mechanisms (Table 1). Such agents principally act through protein kinase C to stimulate cell division (*44*). Details of the combination of different parameters involved in hepatocyte RDS induction by particular agents require further attention.

Examination of early RDS inductive events provides us with a reliable and simple test to determine whether unknown chemicals possess proliferative stimulus potential. The data obtained so far indicate that RDS-positive and Ames-positive agents are hepatocarcinogens in humans and rodents except for 4-nitro-*o*-phenylenediamine and RDS-positive and Ames-negative agents, which are possible hepatocarcinogens. The data show that RDS-negative and Ames-negative agents are probably noncarcinogens in the liver. Thus, use of the two tests in combination is an effective approach for classification purposes.

What is the significance of early hepatocyte RDS induction for hepatocarcinogenesis? The process may lead to cell death for almost all RDS-inductive hepatocytes by means of apoptosis, which involves one of the normal functions of the p53 tumor-suppressor gene (*45*). There are four points to support this: 1) experimental data show that the maximum peak of early RDS is observed approximately 24 to 48 hr after chemical treatments and it disappears soon afterwards (*14,15,23*); 2) it is generally considered that normal p53 function is maintained within 48 hr under conditions of RDS induction; 3) it appears that unequivocal DNA modifications formed by genotoxic hepatocarcinogens greatly contribute to apoptosis (*45*); and 4) there is no direct correlation between hepatocyte RDS incidence and the hepatocarcinogenic potency so that the affected hepatocytes are unlikely to be precursor cell candidates for subsequent hepatic adenomas and carcinomas.

The measured RDS event after a single exposure may simply reflect the early *in vivo* response to chemical exposure, which is likely to be immediately followed by homeostasis. In 2-year animal bioassays, however, it is likely that such cell division occurs continuously and repeatedly at low levels in target issues. As a result, affected cells, apoptosis-resistant cells, will be expected to persist and play a role in the generation of malignant tumor cells over long application times.

Differences between Nongenotoxic Carcinogens and Tumor Promoters

Development of tumors is likely to require at least two initial steps: equivocal DNA modifications and nonprogrammed cell division. The mechanisms underlying carcinogenesis are widely understood to involve multistage, initiation, promotion, and progression processes. Experiments using two-stage animal models have supported the terminology "tumor-initiating" and "tumor-promoting" agents, leading to confusion. Pure tumor promoters have been characterized as not only incomplete carcinogens but also as nongenotoxicants.

Hildebrand et al. (*46*) and Perera (*47*) have indicated that the term "tumor promoters" should be limited to the discussion of two-stage model systems in which tumor development is examined after the application of an initiating agent. The term "nongenotoxic carcinogen" should be used to designate an Ames-negative agent that is capable of causing the development of malignant tumors in 2-year bioassays when animals are exposed to that agent alone. Definite differences between nongenotoxic carcinogens and tumor promoters are not likely to be established; therefore, confusion in the application of terminology will remain.

Tumor promoters are often considered to be carcinogenic when they test positive in 2-year animal bioassays, which leads to the interpretation that tumor promoters are equal to nongenotoxic carcinogens. These nongenotoxic carcinogens are some-

times defined in terms of their carcinogenic potency in a range of animal species, strains, sexes, tissues, or organs, in contrast to the more limited promoter case. Experimental data for nongenotoxic carcinogens tend to show that the two groups are equivalent in practice, and tumor promoters may cause cell proliferation that is tissue or organ specific in animals (*48*). A representative nongenotoxic carcinogen, benzene, which is a rodent carcinogen as well as a human carcinogen, induces tumors in a wide range of animal species and strains in both sexes and in many tissues and organs (*4*). It is questionable, therefore, whether all nongenotoxic carcinogens are simply tumor promoters.

The confusion in use of the terms "nongenotoxic" and "tumor promoters" principally occurs when the cause of human cancers is considered. Current understanding suggests that the capacity of chemicals to cause cell proliferation is more important than the initiating effects of the chemicals. As far as the occurrence of human cancers is concerned, it may not be necessary to make a strict distinction between nongenotoxic carcinogens and tumor promoters as responsible agents. In the experimental field, however, a strict discrimination is always needed for regulatory authorities to properly assess human cancer risk.

My hypothesis is that hepatocarcinogenicity is due to stimulation of cell proliferation and production of equivocal DNA modifications. While the ability of chemicals to induce hepatocyte proliferative division can be determined in RDS experiments, equivocal DNA modifications are difficult to estimate. Overcoming this problem would be of great assistance in assessing human cancer risk. After examination of *in vitro*/*in vivo* DNA-binding adducts caused by more than 200 different DNA-binding agents, Hemminki (*49*) reported on the relationship between ultimate DNA adducts and malignant tumor development. A clear understanding awaits comprehensive *in vivo* DNA-binding data and sufficient quantitative results with regard to dose dependency of response.

For example, DNA adducts have not yet been demonstrated for the representative human carcinogen benzene. The microsomal oxidation of benzene to phenol and of phenol to catechol and hydroquinone are known to be major pathways in the metabolism of benzene. The nature of the ultimate carcinogenic metabolite however has not been resolved, although benzene oxide, catechol, hydroquinone, and benzoquinone have each been proposed as important contributors to its carcinogenicity (*50*). Of the benzene metabolites reported by Leanderson and Tagesson (*51*), catechol and hydroquinone are known to form 8-hydroxydeoxyguanosine *in vitro* (*52*). Thus, determination of equivocal DNA modifications requires further investigation because it is extremely important for distinguishing so-called nongenotoxic carcinogens from tumor promoters.

In the interpretation of increased RDS events, a key point is whether it is possible to distinguish nongenotoxic carcinogens from tumor promoters. The difference is based on whether the chemical possesses the ability to cause equivocal DNA modifications (e.g., oxidative DNA adducts). With regard to cell proliferation induced by tumor promoters, we can speculate that inductive RDS events involve the cascade-pathway protein kinase C (*44*) without equivocal DNA modifications, and such cell proliferative conditions might themselves never lead to tumorigenesis. Nongenotoxic carcinogens, on the other hand, that produce equivocal DNA modifications (e.g., oxidative DNA adducts) were exemplified by 8-hydroxydeoxyguanosine (*52*). Such DNA adducts are considered to never cause genotoxic events in the standard Ames test, but they might contribute to disruption of normal cell division, although any causative significance for oxidative DNA adducts in cell proliferation remains to be proven.

Data on formation of oxidative DNA adducts have been obtained for several carcinogens in both *in vivo* and *in vitro* experiments (*53*). With hepatocarcinogens and experimental animal exposure *in vivo*, at least four agents, [DEHA and DEHP (*54*), 2-nitropropane (*55*), and polychlorinated biphenyls (*56*)] have been shown to cause 8-hydroxydeoxyguanosine in hepatocyte DNA in rats; of these agents, DEHP and DEHA also increased hepatocyte RDS events (Table 1). Thus, oxidative DNA stress might lead to development of hepatocarcinogenesis in cooperation with cell proliferation.

Some of the nongenotoxic noncarcinogens examined also induced hepatocyte RDS events (Table 1). Such false-positive RDS events might be due to a tumor-promoting action on hepatocytes *in vivo*. As indicated by Ledda-Columbano et al. (*39*), differences between nongenotoxic hepatocarcinogen-induced and tumor promoter-induced or mitogen-induced cell proliferation might be distinguished by analyzing the nature of overexpression of proto-oncogenes during early hepatocyte RDS induction. This approach might similarly be important to distinguish nongenotoxic carcinogens from tumor promoters.

A representative promoter without carcinogenic potency, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was found to be carcinogenic in a long-term study when it was repeatedly applied to the skin of BALB/c mice (*57*). Proliferation of keratinocytes is known to be controlled by TGF-β_1 (*40*), and hepatocytes respond to HGF (*41,42*). In addition, keratinocytes in the normal skin of adults are always present as immature, intermediate, and mature cells; hepatocytes in the normal liver of adults are a homogeneous population of mature, differentiated cells. In considering these backgrounds, cell-cycle control mechanisms of keratinocytes probably differ from those of hepatocytes. Namely, skin carcinogenesis by TPA might be due to chronic cell proliferation involving immature keratinocytes that may be more susceptible to cell-cycle checkpoint disruption than mature cells, leading to genetic instability and therefore skin carcinogenesis.

The hypothesis that tumor promoters cause cell proliferation without equivocal DNA modifications may therefore be limited to mature cell cases (e.g., hepatocytes and renal tubule cells). This is of interest in view of the finding that almost all existing nongenotoxic carcinogens induce malignant tumors in the liver and kidney (*1,4,5*).

Genotoxic Noncarcinogens

Genotoxicity tests have been principally performed with bacteria (the Ames test), mammalian cell lines (*in vitro* chromosome aberration test), and hematopoietic cells (*in vivo* mouse micronucleus test). The existence of genotoxic noncarcinogens is considered to be a reflection of the properties of the biological indicator cells used in established genotoxicity tests. Each biological indicator cell is independently capable of progressing through the cell cycle and dividing, but this is not necessarily the case for cells in tissues, such as hepatocytes and renal tubular epithelial cells. This is probably due to the functions of gap junctions (*58–60*).

The cells used in genotoxicity tests are far more susceptible to conversion of genotoxic events to fixed mutations than cells existing in tissues. The applied systems have a number of defects in other areas, e.g., overapplication of drug-metabolizing enzymes, overdoses, and a relative lack of a detoxication process *in vitro*. Application of new genotoxicity tests that use mammalian cells with functioning gap junctions and a normal complement of enzymes is thus needed to prevent false-positive data for genotoxic noncarcinogens in the future.

In this respect, Cunningham et al. (*27*) reported that, although 2,4- and 2,6-diaminotoluene analogs were equally genotoxic for the Ames test, only 2,4-diaminotoluene was hepatocarcinogenic and

increased hepatocyte RDS induction (Table 1). Therefore, they argued that induction of liver tumors is likely to require both genotoxic action and stimulative potential for cell proliferation.

In addition, Goldsworthy et al. (*8*) reported interesting data on hepatocyte *lacI* genotoxic events and hepatocyte proliferation in transgenic mice using two alkylating agents, *N*-dimethylnitrosamine as a representative hepatocarcinogen and methylmethane sulfonate as a nonhepatocarcinogen. Both chemicals are known to be positive in *in vivo* mouse hepatocyte unscheduled DNA synthesis tests (*61*) as well as in the three types of genotoxicity tests. The data showed that *N*-dimethylnitrosamine caused *lacI* genotoxic events and hepatocyte proliferation in hepatocytes *in vivo*, whereas methylmethane sulfonate induced neither.

These findings also provide us with evidence that hepatocytes in tissue *in vivo* need cell proliferation for genotoxic lesions to become fixed and carcinogenesis to result. Thus, determination of proliferative response is considered to be most appropriate to predict hepatocarcinogenicity.

Relationship between Cell Proliferation and Chemical Hepatocarcinogenesis

Nongenotoxic carcinogens and tumor promoters can also act to clonally expand spontaneously occurring, initiated cells. Although clonal expansion by both types of agents may be generally accepted as contributory to development of tumors, at present it is unlikely to be accepted as a theory of tumor development for the reasons described below.

Ward et al. (*62*) reasoned that, if the rate of spontaneously occurring, initiated cells is similar for each specific tissue, then more spontaneous cancers should occur in larger organs. Liu et al. (*63*) reported that HGF inhibited proliferation in glutathione *S*-transferase placental form-positive rat hepatocytes (putative preneoplastic foci cells) induced by *N*-diethylnitrosamine, whereas it stimulated cell division in nonlesion areas. Moreover, Schulte-Hermann et al. (*64*) claimed that putative preneoplastic foci cells of rat livers exhibit approximately 10-fold higher rates of apoptosis than normal hepatocytes. These points must be taken into account in any explanation based on clonal expansion. With regard to carcinogenesis by genotoxic and nongenotoxic carcinogens, the theory of genetic instability, which involves disruption of growth arrest checkpoints, is now considered to be of great advantage to understanding mechanisms of action.

The following summary of how cell proliferation might act in carcinogenesis takes into account recent information. First of all, an imbalance of the deoxynucleotide pool occurs (*65,66*), stimulating dihydrofolate and DNA polymerase α, for example. In the first stage of cell proliferation, this imbalance might be triggered by some factors, such as steroid-superfamily receptor–ligand complexes or HGF–receptor– ligand complexes, as described above. Of these factors, some are known to contribute to overexpression of some proto-oncogenes, which leads to further progression of nonprogrammed cell proliferation (*34–36*).

In the second stage, appreciation of the role of sustained cell proliferation in the carcinogenesis process requires a comprehensive understanding with regard to methylation status (*67–69*). Namely, a chronically maintained high rate of cell division can give rise to an imbalance in normal DNA methylation levels involving 5-methylcytosine (*67–70*), leading to hypermethylation or hypomethylation of intracellular DNA. Alteration of DNA methylation levels may directly cause genetic instability that can result in spontaneous DNA changes (C to T transitions), which are a type of equivocal DNA modifications.

In the third stage, when genes responsible for controlling normal DNA replication such as p53 (*71*) become inactivated, nonprogrammed cell division can progress without G_1 arrest involving the repair of DNA alteration (*68–70,72–75*). Thus, disruption of cell-cycle control check points increases nonprogrammed cell proliferation with elevated genetic instability, leading to the possibility of malignant tumor cells arising in the future. Namely, in genetic instability theory, an abnormality of p53 functions is considered a key factor in resolving the relationship between cell proliferation and chemical carcinogenesis.

A number of recent studies have provided detailed understanding of the complexity of cell-cycle control mechanisms. With regard to the initiation of programmed cell proliferation that Taya (*76*) reviewed, the normal *RB* tumor-suppressor gene plays an extremely important role (*77*). When pRB is phosphorylated at the G_1/S border with active cyclin-dependent kinase (Cdk)–G_1 cyclin complexes, its linked transcription factor (E2F) (*78*) is released, which activates genes relating to progression of cell proliferation. Thus, phosphorylation involving Cdk-G_1 cyclin complexes is promoted by the products of proto-oncogenes (*myc*, *ras*, etc.) and is inhibited by products of tumor-suppressor genes (p15, p16, p27, etc.), thus controlling normal cell division by means of the actions of "brakes and accelerators." It has been argued that the mechanisms of normal cell division are disrupted in carcinoma cells (*76*).

With regard to inductive RDS events, namely nonprogrammed cell proliferation induced by chemicals, I have proposed that the initiation step requires biological stimuli that may give rise to an imbalance in the normal DNA methylation status. There are a number of ways in which this might also trigger unscheduled RDS. Taya (*76*) claimed that cell division is also accelerated when tumor-suppressor gene products (e.g., p15, p16, p27) are directly inactivated by exogenous chemicals. The DNA methylation status is affected by any imbalance between DNA methyltransferase and its demethylase activities in target cells (*69*). It needs to be clarified whether genotoxic or nongenotoxic carcinogens might induce elevated activity of either DNA methyltransferase or its demethylase. DNA methylation processes are known to require choline and methionine; by administering a diet deficient in both chemicals, hepatocarcinomas can be induced, indicating a role for hypomethylation of hepatocyte DNA (*79*). An imbalance in the DNA methylation status may thus exert effects without the necessity of sustained cell proliferation.

Finally, hepatocarcinogenesis should be considered from the standpoint of two events that occur with the lesion progression process and increasing age in experimental animals. In 2-year animal bioassays, it appears that progressively more malignant clones are repeatedly created from background altered populations. Also, with increasing age, the normal function of tumor-suppressor genes tends to gradually and spontaneously disrupt various cell types. By means of both continuous processes, apoptosis-resistant, semi-abnormal hepatocytes, which can go through the first gate of the pathway to tumors with accumulated genetic instability, may be selected. Such genetic instability will contribute to stepwise disruption of oncogenes and some tumor-suppressor genes. When tumor-suppressor genes lose their normal function, apoptosis-resistant abnormal hepatocytes may be able to go through the next gates leading to malignancy. This hypothesis is based on the occurrence of apoptosis-resistant abnormal hepatocytes and is supported by the data of Roberts et al. (*80*), who reported that the majority of the hepatocytes generated during chemical-induced hyperplasia were protected from apoptosis during liver regression. In conclusion, risk assessment of chemical agents should focus on control of early cell proliferation *in vivo*; this present short-term test is available for this purpose.

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